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Crosslinking of CD44 on human osteoblastic cells upregulates ICAM-1 and VCAM-1

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Abstract **Cell adhesion of osteoblasts and osteoclastic precursors of hematopoietic origin is a prerequisite for osteoclast maturation. We have investigated the relevance of osteoblast–matrix binding and regulation of adhesion molecules to this process. Human osteoblastic cells highly expressed CD44, a major receptor for hyaluronan present in the surrounding bone matrix. Crosslinking of CD44 on osteoblastic cells with specific antibodies augmented the expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. Hyaluronan, the major ligand of CD44, also up-regulated ICAM-1 expression. Stimulation of CD44 on osteoblastic cells amplified their adhesion to monocytic cells through ICAM-1 and VCAM-1. These results suggest that such cross-talk among distinct adhesion molecules may be relevant to bone metabolism, including osteoclastogenesis.**

Key words: Osteoblast; Osteoclast; CD44; Hyaluronan; Intercellular adhesion molecule-1; Vascular cell adhesion molecule-1

1. Introduction

The definition of the molecular basis of cell adhesion and its importance in cell–cell and matrix interactions has progressed during the last decade. Adhesion molecules like integrins (LFA-1, VLA-4), the immunoglobulin superfamily (intercellular and vascular cell adhesion molecules (ICAM-1, VCAM-1), and CD44 play major roles in cell–cell, cell–ECM (extracellular matrix) adhesion. Recent reports demonstrate that certain adhesion molecules play a role as 'signal transmitting molecules' as well as their adhesive function. We have previously reported that ICAM-1 on rheumatoid synoviocytes induces cytokine gene transcription via nuclear factor activation and that stimulation of β1 integrin down-regulates ICAM-1 of lung cancer cells through focal adhesion kinase [1,2].

CD44, known as a receptor for hyaluronan, is one of the adhesion molecules that are involved in signal transmission and regulating cellular function. Stimulation of CD44 with monoclonal antibodies (mAbs) or hyaluronan activates T cells, or induces cytokine or chemokine release from monocytes [3,4]. We have previously reported the up-regulation of VCAM-1, Fas and production of interleukin-6 after CD44 stimulation by hyaluronan on rheumatoid arthritis (RA) synovial cells. Stimulation of CD44 on RA synovial cells augmented their adhesion to T cells via the VCAM-1/VLA-4 pathway. [5–7]. These insights have prompted us to investigate whether CD44 is ubiquitously involved in signal transmission, and CD44–ECM adhesion plays an important role in regulating the expression of other adhesion molecules, such as ICAM-1 and VCAM-1. Osteoblasts are one of the candidates for these investigations as osteoblasts are surrounded by bone matrix including hyaluronan, and adhesion of osteoblasts to osteoclastic cells through the ICAM-1, VCAM-1 pathway is crucial for osteoclastogenesis [8]. In addition, the precise role of CD44 on osteoblasts largely remains unknown.

We here document that CD44-mediated signaling on osteoblastic cells induces the expression of ICAM-1 and VCAM-1, leading to augmented adhesion of osteoblastic cells to the monocytic cell line U937 mediated by these molecules. We propose a new regulatory mechanism for bone metabolism regulated by CD44–ECM interaction.

2. Materials and methods

2.1. Preparation of human osteoblastic cells

Osteoblast-like cells were purified from metaphyseal trabecular bone in the proximal femur of four osteoarthritis patients during total hip arthroplasty by the established procedures of Beresford et al. [9]. Briefly, after removing pieces of cortical bone, articular cartilage, and soft connective tissue, the fragments were cut into small pieces and washed extensively. The bone explants were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (FCS, Gibco) in tissue culture plates. When cell monolayers were confluent after 6–8-week culture, the explants were removed and the cells were replated and incubated, which resulted in new cellular outgrowth and eventually a confluent monolayer of cells which possessed characteristics of the osteoblastic phenotype. Cells were used at the third to seventh passage.

2.2. Cell culture

The human monocytic lymphoma cell line U937 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cell lines were grown in DMEM with 10% heat-inactivated FCS.

2.3. Reagents and mAbs

Fragmented and native hyaluronan were kindly donated by the Tokyo Research Institute of Seikagaku Co. The following mAbs were used as purified immunoglobulin (Ig): major histocompatibility class (MHC) class I mAb W6/32, anti-glycophorin mAb 10F7, CD11a (LFA-1α) mAb TS1/22 (ATCC), control mAb Thy1.2, CD106 (VCAM-1) mAb (Becton Dickinson, San Jose, CA, USA), CD44 blocking mAb BU75 (Ancell, Bayport, MN, USA), CD44 mAb NIH44-1, CD54 (ICAM-1) mAb 84H10, CD49d (VLA-4) mAb NIH49d-1 (gifts from Dr. S. Shaw, Bethesda, MD, USA), and CD106 mAb 2G7 (gift from Dr. W. Newman, Rockville, MD, USA).

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2.4. Stimulation of CD44

Cells were cultured until subconfluence and then incubated with NIH44-1 mAb (10 μg/ml) for 30 min at 37°C. After washing the cells three times, 1 μg/ml of goat anti-mouse IgG-Fc was added as the second Ab for CD44 crosslinking. The cells were also incubated with fragmented or native hyaluronan (0.1 μg/ml) for 6 h at 37°C.

2.5. Flow microfluorometry

Staining and flow cytometric analysis of osteoblastic cells were carried out by standard procedures as already described using a FACScan (Becton Dickinson, Mountain View, CA, USA). Briefly, cells (2×10^5) were incubated with specific mAbs and subsequently fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG Ab, or incubated with phycoerythrin (PE)-conjugated CD54 (ICAM-1) mAb or FITC-conjugated CD106 (VCAM-1) mAb at saturating concentrations in FACS medium consisting of Hanks' balanced salt solution (Nissui, Tokyo, Japan), 0.5% human serum albumin (Yoshitomi, Osaka, Japan), and 0.2% NaN_3 (Sigma Aldrich) for 30 min at 4°C. After three washes in FACS medium, the cells were analyzed with FACScan. Amplification of the mAb binding was provided by a three-decade logarithmic amplifier. Quantification of the cell surface antigens on one cell was performed using beads, QIFKIT (Dako Japan, Kyoto, Japan).

2.6. Adhesion assay

The adhesion assay was performed as previously described [8,10,11]. Briefly, osteoblastic cells were applied to 24-well culture plates (Nunc, Roskilde, Denmark) and cultured in DMEM with 10% heat-inactivated FCS. U937 cells were labeled with sodium ^{51}chromate (Dupont NEN). 1×10^6 U937 cells in 1 mM phorbol myristate acetate (PMA, Sigma Aldrich) in the presence or absence of relevant blocking mAb (10 μg/ml) were added and the cells incubated at 37°C for 30 min in phosphate-buffered saline with 0.5% human serum albumin: non-adherent cells were washed off completely. Well contents were lysed with 1% Triton X-100 (Sigma Aldrich), and γ-emissions of adherent wells were determined.

2.7. Statistical analysis

Significant differences among groups within each experiment were determined by ANOVA, followed by post-hoc Scheffe's *F*-test.

3. Results

3.1. Human osteoblastic cells highly express CD44, ICAM-1

First, we assessed the cell surface adhesion molecules on purified human osteoblastic cells using FITC- or PE-conjugated mAbs by FACScan. A high level of CD44 expression, a major receptor for hyaluronan, was detected on basal osteoblastic cells (Fig. 1). About half of the osteoblastic cells spontaneously expressed ICAM-1 without stimulation. However, unlike CD44 or ICAM-1, the basal expression of VCAM-1 was almost negative on osteoblastic cells without stimulation. These results demonstrate that osteoblastic cells express adhesion molecules like CD44 and ICAM-1 in a stimulation-independent manner.

3.2. CD44 crosslinking up-regulates ICAM-1 and VCAM-1 expression on osteoblastic cells

To characterize the function of CD44, we crosslinked cell surface CD44 molecules on osteoblastic cells using a specific anti-CD44 mAb and a second crosslinker Ab. The surface expression of ICAM-1 and VCAM-1 was measured by FACScan. After CD44 crosslinking on osteoblastic cells, we observed a strong up-regulation of ICAM-1 (Fig. 1B,C). VCAM-1 was also up-regulated after CD44 stimulation with a specific mAb (Fig. 1D,E). Next, we quantitated the change of expression level of ICAM-1 and VCAM-1 after CD44 stimulation using FACScan and QIFKIT standard beads. As shown in Fig. 2, ICAM-1 was up-regulated two- to four-fold after CD44 crosslinking, but not by crosslinking with control anti-MHC class I mAb. VCAM-1 was almost negative on basal osteoblastic cells, however, 8–20-fold up-regulation of VCAM-1 molecules was observed after CD44 crosslinking. Though the alteration of ICAM-1 molecules after CD44

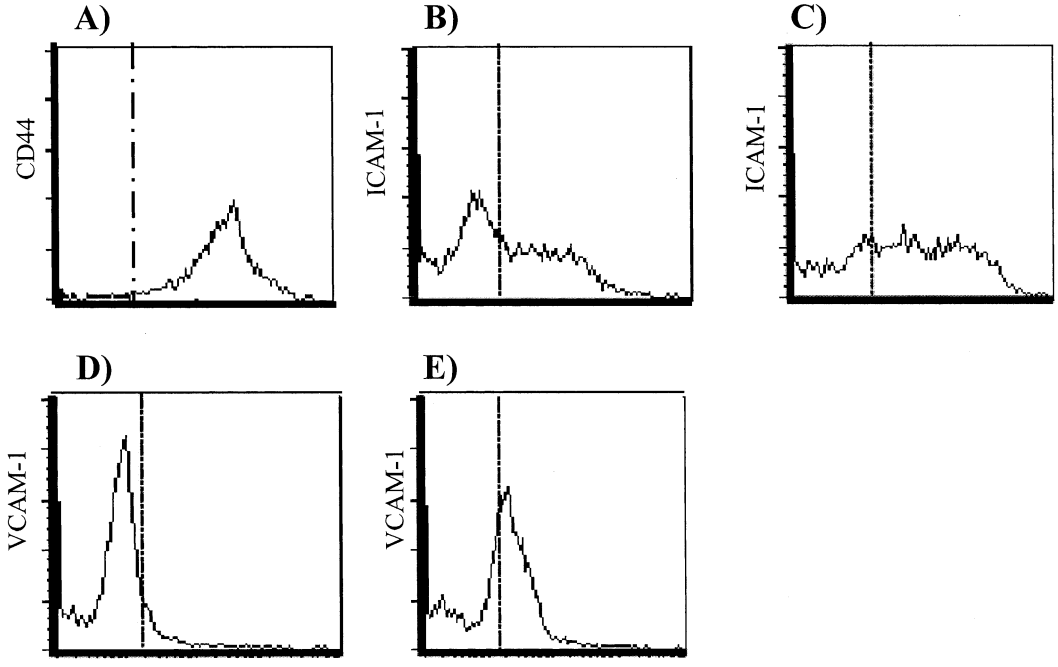

Fig. 1. Phenotypic analysis and up-regulation of ICAM-1 and VCAM-1 by CD44 crosslinking on osteoblastic cells. Bone samples were obtained from four osteoarthritis patients during total hip arthroplasty and osteoblastic cells were isolated. Purified human osteoblastic cells were stained with CD44 mAb NIH44-1 (A), ICAM-1 mAb 84H10 (B,C), VCAM-1 mAb 2G7 (D,E). Osteoblastic cells were incubated in medium (B,D) or crosslinked with anti-CD44 mAb NIH44-1 (C,E) at a concentration of 10 μg/ml for 6 h. Flow cytometric analyses of ICAM-1 and VCAM-1 expression were performed using FACScan. Histograms are representative data from four different donors.

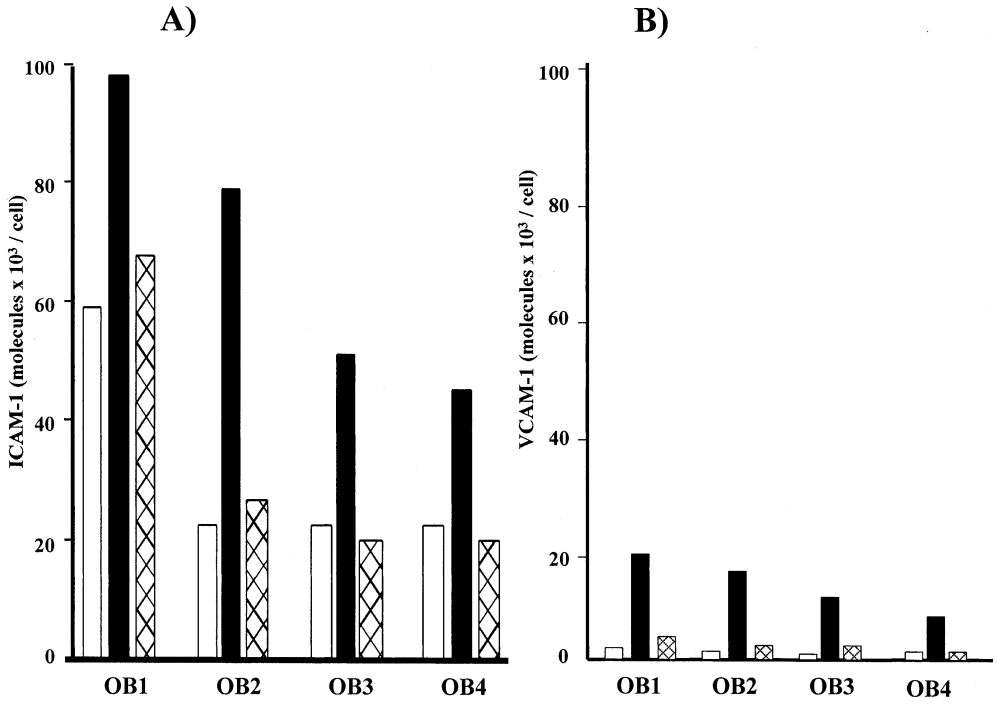

Fig. 2. Quantification of ICAM-1 and VCAM-1 increased by CD44 crosslinking on osteoblastic cells. Purified human osteoblastic cells were crosslinked with anti-CD44 mAb NIH44-1 or control anti-MHC class I mAb W6/32 at a concentration of 10 μg/ml for 6 h and the expression of ICAM-1 (A) and VCAM-1 (B) was analyzed by FACScan. Cell surface molecules on a single cell were quantified by standard beads QIFKIT, after stimulation with control medium (open bars), anti-CD44 mAb (solid bars) or anti-MHC class I (hatched bars). Shown are representative data of three different experiments from four different donors (designated OB1 to 4).

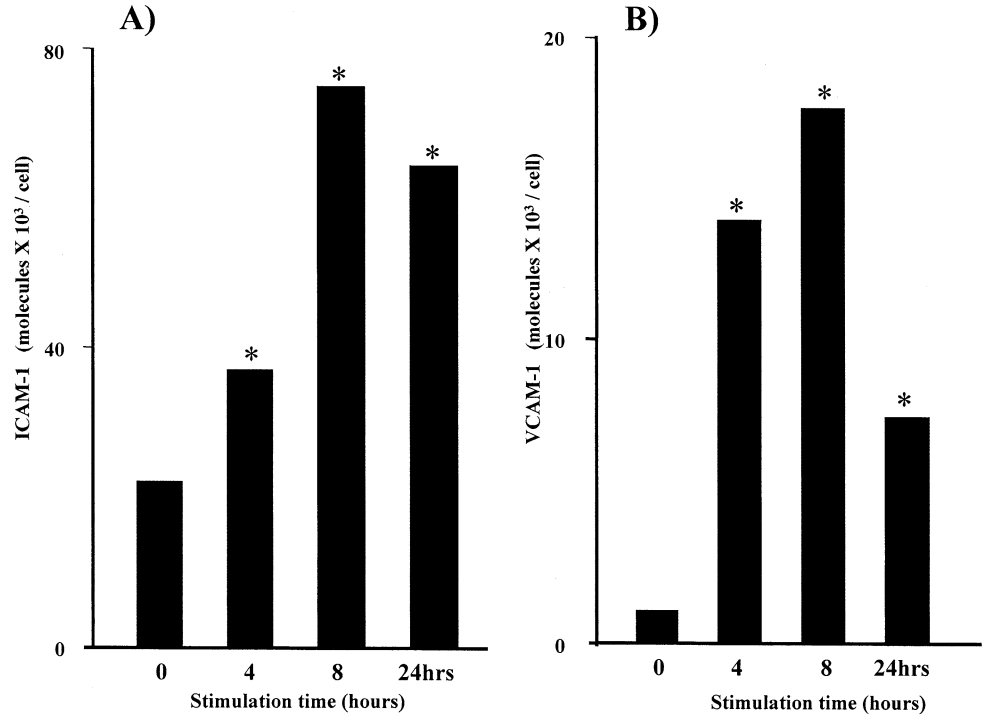

Fig. 3. Kinetic study of ICAM-1 and VCAM-1 up-regulation after CD44 stimulation. Human osteoblastic cells were stimulated with 10 μg/ml of anti-CD44 mAb for the indicated duration. The expression of ICAM-1 and VCAM-1 was analyzed by FACScan. Shown is the quantification of the cell surface antigens on a single cell, calculated by QIFKIT, of representative data among four different experiments. $*P < 0.05$ compared with control.

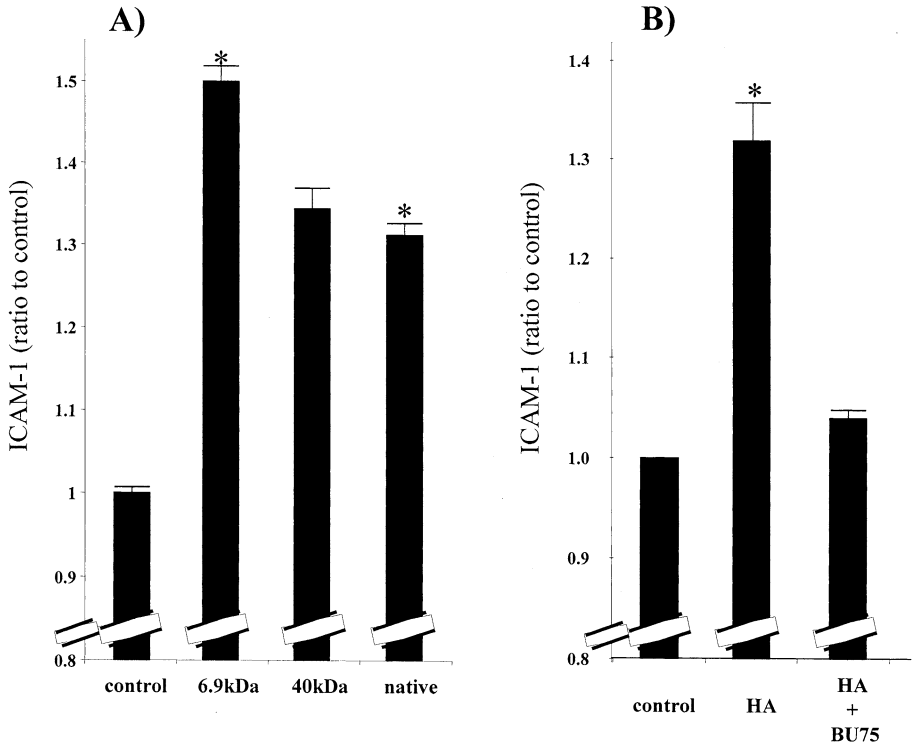

Fig. 4. Upregulation of ICAM-1 on osteoblasts by hyaluronan. Osteoblastic cells were incubated with 100 ng/ml of fragmented (6.9 kDa and 40 kDa) or full-length hyaluronan for 6 h (A). Osteoblastic cells were stimulated with fragmented 6.9-kDa hyaluronan in the presence or absence of the anti-CD44 mAb BU75 (B). Expression of ICAM-1 was analyzed by FACScan. After quantification of the cell surface antigens on a single cell, calculated by QIFKIT, the ratio to the quantity of ICAM-1 on unstimulated cells was obtained, among four different experiments. Data are presented as mean ± S.D. $*P < 0.05$ compared with control.

crosslinking was smaller than that of VCAM-1, ICAM-1 exceeded VCAM-1 by 5–10-fold in the total number of molecules expressed on the osteoblastic cell surface.

Time course experiments showed that ICAM-1 expression on osteoblasts reached maximum levels (approximately a four-fold increase compared to unstimulated cells) in 8 h after CD44 crosslinking, lasting until 24 h of incubation (Fig. 3A). VCAM-1 was markedly up-regulated in 4 h, reaching its maximum level in 8 h after CD44 crosslinking (Fig. 3B). Comparing the induction pattern of these two molecules, VCAM-1 had a tendency to decrease more rapidly than ICAM-1. These results demonstrate that CD44 is a potent inducer of ICAM-1 and VCAM-1 expression on osteoblastic cells.

3.3. *Hyaluronan up-regulates ICAM-1 and VCAM-1 on osteoblasts*

Hyaluronan is known as a major ligand for cell surface CD44. We assessed the biological activity of hyaluronan in ICAM-1 and VCAM-1 expression on osteoblastic cells. Stimulation of CD44 with hyaluronan efficiently up-regulated ICAM-1 on osteoblastic cells (Fig. 4A). In addition, various lengths of hyaluronan fragments were tested for ICAM-1 up-regulation, as the different sizes of hyaluronan are reported to possess different biological effects. Different lengths of hyaluronan, including native hyaluronan, a 40-kDa fragment and a 6.9-kDa fragment, up-regulated ICAM-1 expression. Among them, the fragmented 6.9-kDa hyaluronan most effectively augmented ICAM-1 expression (Fig. 4A). To demonstrate the direct interaction of hyaluronan and CD44, we stimulated osteoblasts in the presence and absence of the anti-CD44 blocking Ab BU75. As shown in Fig. 4B, up-regulation of ICAM-1 induced by fragmented hyaluronan was inhibited by the anti-CD44 mAb. Hyaluronan also augmented VCAM-1 expression, however, this increase was not blocked by mAb BU75 (data not shown). These results indicate that hyaluronan, especially certain fragmented types, appears to be an inducer of ICAM-1 on osteoblastic cells through binding to CD44.

3.4. *CD44 stimulation augments adhesion of osteoblasts to monocytic cells through up-regulation of ICAM-1/VCAM-1*

We investigated whether CD44-induced ICAM-1 and VCAM-1 up-regulation of osteoblastic cells augments their adhesion to the monocytic cell line U937 [12,13]. 10–15% of PMA-pretreated U937 cells bound non-stimulated osteoblastic cells (Fig. 5A). Engagement with control mAb W6/32 did not induce their adhesion and the adhesion was scarcely decreased by the addition of anti-LFA-1 mAb (data not shown). However, a two- to five-fold increase of this binding was observed after crosslinking of CD44 on osteoblastic cells with a specific mAb (Fig. 5A). This increased binding was significantly inhibited in the presence of anti-VLA-4 mAb, and especially by LFA-1 mAb. Also, a binding assay was performed by stimulating osteoblastic cells with a 6.9-kDa fragment of hyaluronan. As shown in Fig. 5B, fragmented hyaluronan efficiently augmented the binding of osteoblastic cells to PMA-pretreated U937 cells, and the binding was inhibited in the presence of anti-VLA-4 mAb or anti-LFA-1 mAb.

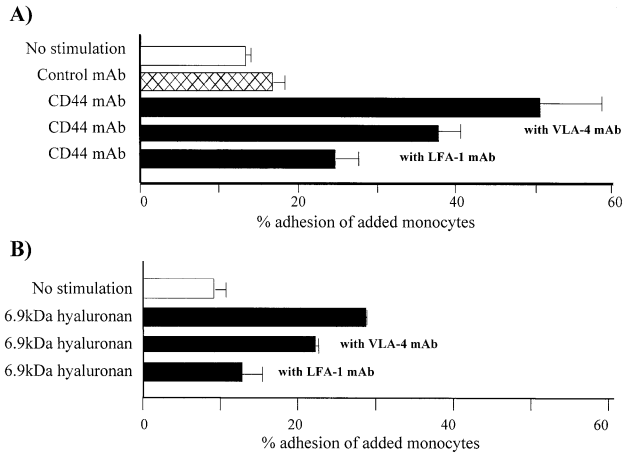

Fig. 5. Adhesion of CD44-crosslinked or hyaluronan-stimulated osteoblastic cells to the monocytic cell line U937. CD44 on osteoblastic cells was engaged with CD44 mAb or control MHC class I mAb with a subsequent second polyclonal Ab (A), or with a 6.9-kDa fragment of hyaluronan (B) for 12 h. ^{51}Chromate-labeled U937 cells were added in the presence of anti-LFA-1 or VLA-4 blocking mAb. Data are expressed as mean percentage and standard deviation of the binding of added labeled cells from triplicate wells, of representative results among five different experiments.

These results demonstrate that CD44 plays an important role in increasing the binding of osteoblastic cells/monocytic cells by up-regulation of adhesion molecules, especially ICAM-1. Augmentation of the adhesion by hyaluronan stimulation suggests that hyaluronan, especially fragmented types, is a biologically important stimulator for CD44, and that might be increasing osteoblast/monocyte binding through the LFA-1/ICAM-1 pathway.

4. Discussion

Adhesion molecules are involved in signaling in two reverse directions in cell–cell and cell–matrix interactions. We here propose that stimulation of the adhesion molecule CD44 per se plays a pivotal role in the amplification of particular adhesion molecules, ICAM-1 and VCAM-1 expression. We deduce this from the following novel findings: (1) CD44 crosslinking on osteoblastic cells up-regulated ICAM-1 and VCAM-1 expression; (2) hyaluronan, the ligand of CD44, also effectively increased ICAM-1, and (3) integrin-dependent adhesion of osteoblastic cells to U937 was markedly increased by stimulating CD44 on osteoblastic cells.

CD44 is a 90-kDa transmembrane glycoprotein widely distributed on the surface of most leukocytes, fibroblasts, keratinocytes, epithelial cells, and osteoblastic cells [14,15]. Our present report strengthens the important role of CD44 as a signal-transmitting molecule, and further suggests the concept that CD44–ECM interaction is ubiquitously playing a pivotal role for regulating the expression of adhesion molecules.

The best known ligand for CD44 is hyaluronan. Hyaluronan is a major extracellular glycosaminoglycan, found in almost all types of ECMs including bone matrix. Several studies have suggested diverse biological activities of hyaluronan depending on its molecular mass. High molecular mass hyaluronan inhibits cellular proliferation of endothelial cells, fibroblasts, and mitogen-stimulated lymphocytes, whereas low molecular mass fragments have stimulatory effects on angiogenesis and cytokine production [3,4,16–20].

We have previously reported that stimulation of RA synovial cells with 6.9-kDa hyaluronan up-regulated VCAM-1, whereas in the present study, the same stimulus up-regulated dominantly ICAM-1 on osteoblastic cells [5]. 6.9-kDa hyaluronan up-regulated Fas on RA synovial cells, while down-regulation of Fas was observed by the same stimulus on a lung cancer cell line [21]. These results suggest that the same molecular mass of hyaluronan can exert different biological effects depending on cell type, and further investigation is required to clarify the underlying mechanism.

The structural material of bone is a composite of organic and inorganic phases. The organic bone matrix component is made of collagen and non-collagenous proteins, such as osteocalcin, osteonectin, proteoglycans, and hyaluronan, which are synthesized by osteoblasts and osteoclasts [22–24]. Hyaluronan is known to localize around the CD44-positive osteoblasts [25], however, the fragmented hyaluronan more potently up-regulated ICAM-1 than full-length hyaluronan. These results suggest that ICAM-1 on osteoblasts is up-regulated when fragmented hyaluronan, but not full-length hyaluronan, is produced nearby. Metastasis of malignant cells to bone might be one of the cases that fragmented hyaluronan is enriched around CD44-positive osteoblasts. Tumor metastasis to bone is known to facilitate bone resorption. In the case of breast cancer, significant increases of hyaluronidase levels, considered to be derived from tumor cells, are observed in metastatic sites compared to the primary tumor [26]. These results suggest that locally produced fragmented hyaluronan may stimulate CD44 on osteoblasts, and facilitate the binding of osteoblast/osteoclast precursor cells via up-regulated ICAM-1, leading to facilitation of osteoclastogenesis and bone resorption.

Based on the findings presented here, we propose that CD44 is a ubiquitously regulating cellular function, i.e. we observed that stimulation of CD44 by crosslinking or ligation with hyaluronan induces up-regulation of ICAM-1 and VCAM-1 on osteoblastic cells. These interactions of CD44 and hyaluronan may play a pivotal role in bone metabolism, including osteoclastogenesis.

References

[1] Koyama, Y., Tanaka, Y., Saito, K., Abe, M., Nakatsuka, K., Morimoto, I., Auron, P.E. and Eto, S. (1996) J. Immunol. 157, 5097–5103.
[2] Yasuda, M., Tanaka, Y., Tamura, M., Fujii, K., Sugaya, M., So, T., Takenoyama, M. and Yasumoto, K. (2001) Cancer Res. 61, 2022–2030.
[3] Webb, D.S., Shimizu, Y., Van Seventer, G.A., Shaw, S. and Gerrad, T.L. (1990) Science 249, 1295–1297.
[4] McKee, C.M., Penno, M.B., Cowman, M., Burdick, M.D., Strieter, R.M., Bao, C. and Noble, P.W. (1996) J. Clin. Invest. 98, 2403–2411.
[5] Fujii, K., Tanaka, Y., Hubscher, S., Saito, K., Ota, T. and Eto, S. (1999) J. Immunol. 162, 2391–2398.
[6] Fujii, K., Tanaka, Y., Hubscher, S., Saito, K., Ota, T. and Eto, S. (1999) Lab. Invest. 79, 1439–1446.
[7] Fujii, K., Fujii, Y., Hubscher, S. and Tanaka, Y. (2001) J. Immunol. 167, 1198–1203.
[8] Tanaka, Y., Morimoto, I., Nakano, Y., Okada, Y., Hirota, S., Nomura, S., Nakamura, T. and Eto, S. (1995) J. Bone Miner. Res. 10, 1462–1469.
[9] Beresford, J.N., Gallagher, J.A., Poser, J.W. and Russell, R.G. (1984) Metab. Bone Dis. Relat. Res. 5, 229–234.

[10] Tanaka, Y., Adams, D.H., Hubscher, S., Hirano, H., Siebenlist, U. and Shaw, S. (1993) Nature 361, 79–82.
[11] Tanaka, Y., Maruo, A., Fujii, K., Nomi, M., Nakamura, T., Eto, S. and Minami, Y. (2000) J. Bone Miner. Res. 15, 1912–1923.
[12] Formigli, L., Orlandini, S.Z., Benvenuti, S., Masi, L., Pinto, A., Gattei, V., Bernabei, P.A., Robey, P.G., Collin, O.P. and Brandi, M.L. (1995) J. Cell Physiol. 162, 199–212.
[13] Gattei, V., Aldinucci, D., Quinn, J.M., Degan, M., Cozzi, M., Perin, V., Iuliis, A.D., Juzbasic, S., Improta, S., Athanasou, N.A., Ashman, L.K. and Pinto, A. (1996) Cell Growth Differ. 7, 753–763.
[14] Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C.B. and Seed, B. (1990) Cell 61, 1303–1313.
[15] Lesley, J., Hyman, R. and Kincade, P.W. (1993) Adv. Immunol. 54, 271–335.
[16] West, D.C., Hampson, I.N., Arnold, F. and Kumar, S. (1985) Science 228, 1324–1326.
[17] Goldberg, R.L. and Toole, B.P. (1987) Arthritis Rheum. 30, 769.
[18] Anastassiades, T. and Robertoson, W. (1984) J. Rheumatol. 11, 729.
[19] Forrester, J.V. and Balazs, E.A. (1980) Immunology 40, 435.
[20] Alhgren, T. and Jarstrand, C. (1984) J. Clin. Immunol. 4, 246.
[21] Yasuda, M., Tanaka, Y., Fujii, K. and Yasumoto, K. (2001) Int. Immunol. 13, 1309–1319.
[22] Rodan, G.A. (1998) J. Cell Biochem. 30–31 (Suppl.), 55–61.
[23] Midura, R.J., Evanko, S.P. and Hascall, V.C. (1994) J. Biol. Chem. 269, 13200–13206.
[24] Nakamura, H. and Ozawa, H. (1994) J. Bone Miner. Res. 9, 1289–1297.
[25] Nakamura, H. and Ozawa, H. (1996) J. Bone Miner. Res. 11, 1715–1722.
[26] Bertrand, P., Girard, N., Duval, C., d'Anjou, J., Chauzy, C., Menard, J.F. and Delpech, B. (1997) Int. J. Cancer 73, 327–331.